

Application
for
United States Letters Patent

To all whom it may concern:

Be it known that

B. Jack Longley

have invented certain new and useful improvements in
Uses of Transgenic Animals for Human Skin Diseases

of which the following is a full, clear and exact description

USES OF TRANSGENIC ANIMALS FOR HUMAN SKIN DISEASES

The invention described herein was made in the course of work done under Grant Nos. 1 R29 AR 40514-01A1, 5 P30 5 041942 and 1-RO1-AR43356-01A2 from National Institutes of Health. Therefore, the United States Government has certain rights in this invention.

Throughout this application, various publications are 10 referenced by author and date. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in 15 order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Background of the Invention

20 The use of murine model to investigate human cutaneous oncology, immunology and keratinocyte biology is advantageous over the use of human skin for obvious reasons. However, substantial differences exist between 25 human skin and murine skin. In human skin, Stem Cell Factor is produced by epidermal keratinocytes after birth, unlike in normal murine skin. The result of this, among other things, is that melanocytes are present in the interadnexal epidermis in human skin. In contrast, 30 melanocytes in adult murine skin are generally confined to hair follicles, with the exception of rare epidermal melanocytes found in the ears, footpads, and tail (1). A few dermal melanocytes may also be found in mice, mostly in the ears. These differences have compromised the use of the 35 mice as a model system for investigation of human cutaneous biology.

It has been discovered that melanocyte migration and development, as well as the survival of melanocytes and mast cells, are dependent on expression of the kit protein, a receptor tyrosine kinase encoded by the c-kit proto-oncogene (2-6). The ligand for kit, known as stem cell factor (SCF) (also called mast cell growth factor, steel factor, and kit ligand) may be produced locally in human skin by epidermal keratinocytes, fibroblasts, and endothelial cells (7-8). However, definitive studies of SCF production in murine skin have not been reported. Transgenic studies using the SCF gene promoter region and beta-galactosidase as a reporter gene suggest that, unlike in human skin, postnatal murine cutaneous SCF expression is limited to the dermis and hair follicles, and not found in epidermal keratinocytes (9). The difference in SCF expression between human and murine epidermis could explain the difference in melanocyte distribution and other biological phenomena in these two species.

SCF may be produced in two isoforms by alternate splicing of exon 6. One isoform lacks exon 6 encoded sequences and exists predominantly as a membrane-bound molecule. The other isoform contains exon 6 encoded sequences which include a protease sensitive site (10-19). Cleavage at the protease sensitive site causes the release of a soluble, bioactive form of SCF. The membrane-bound and soluble forms of SCF have differential effects on melanocyte precursor dispersal and survival (20) and exogenous soluble SCF may produce cutaneous mast cell hyperplasia and cutaneous hyperpigmentation (21-23). In addition, local high concentrations of soluble SCF have been found in lesions of human cutaneous mastocytosis, a disease characterized by dermal accumulations of mast cells and increased epidermal melanin (7, 8, 24) and in spongiotic dermatitis, a common inflammatory condition of human skin (our unpublished data).

Summary of the Invention

The present invention provides a method of identifying a composition, a compound, or a procedure which can produce a skin response in a subject, comprising: a) administering said composition or compound, or applying said procedure to the transgenic mice which express endogenous epidermal stem cell factor, and b) analyzing the skin of said transgenic mice for response. The present invention also provides a method of identifying a composition, a compound, or a procedure which can reduce or treat skin response in a subject, comprising: a) administering said composition or compound, or applying said procedure to the transgenic mice which express endogenous epidermal stem cell factor and which had been induced to produce a skin response and b) analyzing the skin of said transgenic mice to determine the reduction of skin response, wherein the reduction of skin response indicates that the composition, compound or procedure can reduce skin response.

The present invention further provides a method of identifying a composition, a compound, or a procedure which can reduce radiation damage to the skin of a subject, comprising: a) administering said composition or compound, or applying said procedure to the transgenic mice which express endogenous epidermal stem cell factor, b) subjecting the skin of said transgenic mice and the skin of the control transgenic mice to radiation, and c) analyzing the effects of said composition, compound, or procedure on reducing skin radiation damages. The present invention also provides a pharmaceutical composition for treating human skin diseases, comprising (a) a compound that can treat skin diseases of the transgenic mice which express endogenous epidermal stem cell factor, and (b) a suitable carrier, wherein the compound specifically targets the epidermal stem cell factor or its receptor.

Brief Description of the Figures

Figure 1: Transgene design. Both transgenes used the human keratin 14 promoter and polyadenylation sequences. Transgene one included a rabbit b-globin intron, and transgene two included human growth hormone sequences to provide for stability. Neither the beta globin intron nor the human growth hormone sequences produce protein products.

Figure 2: Increased mast cells in mice expressing epidermal membrane and soluble SCF (transgene one). (a) Numerous mast cells are demonstrated in the superficial dermis of body wall skin of newborn mice bearing transgene one (membrane/soluble SCF), using an immunoperoxidase/ alcian blue technique which stain mast cell granules metachromatically purple. Note the apposition of mast cells (arrowheads) to basilar keratinocytes, the source of SCF. Immunoperoxidase with an anti-S100 antibody in this preparation also demonstrates melanocytes as brown staining cells in the epidermal basilar layers of epidermis and follicles (white arrows). Sebocytes are seen as large, round, lightly S-100(+) cells in the follicular epithelium. Melanin pigment is stained black in this preparation. (b) Immunofluorescence with anti-kit antibodies highlights kit expressing dermal mast cells (arrowheads) in body wall skin of newborn (transgene one membrane/soluble SCF) mouse. (c) Anti-kit antibody immunofluorescence shows mast cells crowded in the papillary dermis and extending into the upper reticular dermis and body wall skin of 21 day old, transgene one

positive mouse, MC, confluent mast cells; arrowheads, individual and small clusters of mast cells; E, epidermis; F, follicles; K, keratin layer. (d) Hematoxylin and eosin-stained sections show mast cells filling the superficial corium in section of tongue from a 21 day old, transgene one positive mouse. The lack of abundant melanocytes and melanophages in this anatomic site allows easy visualization of the mast cells. This histologic picture is identical to that seen in human cutaneous mastocytosis. (e) Alcian blue stained serial section of tongue shows metachromatic granules in mast cells of 21 day old, transgene one positive mouse.

Figure 3: Electron microscopy confirms the presence of melanocytes and mast cells in transgenic mice. (a) Transgene one mouse with membrane/soluble epidermal SCF has numerous dermal mast cells (arrowheads) as well as dermal melanocytes (arrows). Asterisks show the boundary of the dermis and hair follicle. Higher magnification images of mast cell and melanocyte are shown in b and c, respectively. Original magnifications: (a) 2,750, (b) 9,000, (c) 11,750.

Figure 4: Transgenic phenotypes are stable across a wide range of gene expression levels. This figure compares the transgene copy number determined by PCR, with SCF mRNA expression as determined by RNase protection assay, in lines from different founders. The relative density of SCF bands was determined by dividing the mean density of the SCF band by the density of a SCF band derived from an identical aliquot of RNA. Probe templates were 384 bases in length for SCF (40

base pairs of promoter sequence and 342 bases
complimentary to nucleotides 814-1156 of murine
SCF mRNA (5). A beta-actin probe was used as a
control, and to allow standardization between
5 RNA preparations from different mice. The beta-
actin probe length was 310 bases, 227 bases of
which are complementary to murine beta-actin
mRNA. The probe was purchased from Ambion
10 (pTR1-beta-actin-mouse anti-sense control
template). Note the differences between TG2
(4x, 5x, 10x) and TG1 (6x).

Figure 5: Epidermal SCF causes hyperpigmentation of murine
15 skin. (a) Newborn mouse expressing membrane/
soluble SCF (transgene one, left) shows obvious
hyperpigmentation compared to non-transgenic
littermate (right). (b) Transgene two positive
20 mouse overexpressing membrane-bound epidermal
SCF shows a similar phenotype with generalized
hyperpigmentation which is most discernible in
the ventral and hairless areas, and which is
maintained in adult life. Three week old
25 transgenic (left) and non-transgenic littermate
(right)

Figure 6: Intraepidermal melanocytes are increased in
transgenic mice. (a) Tail skin section from 21
day old mouse expressing epidermal
30 membrane-bound SCF (transgene two) shows mild
epidermal hyperplasia and a markedly increased
number of melanocytes, identified as cells
surrounded by clear halos, mostly at the
dermal-epidermal junction. These mice also show
extensive black epidermal melanin pigment
35 (400x). (b) Note the lack of both basilar
melanocytes and epidermal pigment in the skin of
the transgene (-) littermate control mouse (C57

black 6 (400x)). (c) Epidermal melanocytes express kit protein. Immunofluorescence staining with anti-kit antibody and Texas Red labeled secondary antibody demonstrates confluent dendritic cells in the epidermal basalar layer of mice expressing membrane-bound SCF (transgene two arrows). These cells correspond to the S-100 protein (+) basilar dendritic cells seen in Figure 2a. Note two strongly kit positive solitary mast cells in the dermis (arrowheads, 400x). Light staining of dendritic melanocytes can also be seen in the epidermis of transgene one positive mice (please see figure 2b).

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Figure 7: Electron microscopy confirms the presence of epidermal melanocytes in both types of transgenic mice. (a) Electron microscopy shows numerous keratinocytes containing phagocytized melanin granules in the interadnexal epidermis of mice expressing membrane-bound epidermal SCF (3500x). b. Epidermal melanosomes, some marked with large arrows, are present in both keratinocytes and melanocytes. Pre melanosomes, marked with the open arrows, demonstrate the presence of a melanocyte. Note keratinocyte hemidesmosomes (small arrows) which confirm the location of the melanocyte within the epidermis (16, 320x).

Figure 8: Allergic ear swelling is significantly increased in SCF transgenic animals, and is reduced by blocking the SCF receptor with the ACK2 monoclonal antibody. All transgenic mice show increased ear swelling in response to allergic contactants compared to non-transgenic animals ($p \leq 0.0001$), showing that SCF contributes to

5 dermatitis. Similar results are seen with irritant contactants (data not shown). The ear swelling is specifically decreased by the ACK2 monoclonal antibody which blocks the SCF receptor ($p \leq 0.05$) confirming that epidermal SCF plays an active role in cutaneous inflammation.

Figure 9:

10 9a: Our unpublished immunoperoxidase study of inflamed human skin with an anti-human SCF monoclonal anti-body shows soluble epidermal SCF in spongiotic (eczematous) dermatitis, here demonstrated in an epidermal spongiotic vesicle. Human spongiotic dermatitis may be associated with hyperpigmentation. Soluble epidermal SCF is not detected in normal skin with this technique (7), suggesting that epidermal SCF is released in cutaneous inflammatory states.

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20 9b: Irritant dermatitis induced in transgenic mice by Croton oil is characterized histologically by spongiotic (eczematous) dermatitis. The murine dermatitis is shown in a hematoxylin and eosin stained slide because no satisfactory antibody against murine SCF is available to allow demonstration of soluble SCF by immunoperoxidases. However, these results combined with the ACK2 blocking studies shown above implicate epidermal SCF in this phenomenon.

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Detailed Description of the Invention

The present invention provides a method of identifying a composition, a compound or a procedure which can produce
5 a skin response in a subject, comprising: a) administering said composition or compound, or applying said procedure to the transgenic mice which express endogenous epidermal stem cell factor, and b) analyzing the contacted skin for response.

10 In one embodiment of the method, the composition or compound can be administered orally or by injection.

15 In another embodiment of the method, the composition or compound can be administered topically by contacting the composition or compound with the skin of the transgenic mice.

20 In another embodiment of the method, the procedure is not previously known.

In another embodiment of the method, the procedure is identified by the method.

25 In another embodiment of the method, the procedure is DNA vaccination.

30 In this invention, the skin response may be induced. This skin response includes but is not limited to inflammation, tanning, melanoma, carcinoma or hyperpigmentation.

In another embodiment of the method, the composition may be cosmetics, medications or skin care products.

35 In another embodiment of the method, the composition or compound is not previously known.

In yet another embodiment of the method, the composition or compound is identified by the method.

5 In a further embodiment of the method, a mixture is produced for producing a skin response comprising an effective amount of the composition or compound identified by the method and a suitable carrier.

10 The present invention also provides a method of identifying a composition, a compound, or a procedure which can reduce or treat skin response in a subject, comprising: a) administering said composition or compound, or applying said procedure to the transgenic mice which express endogenous epidermal stem cell factor and which had been 15 induced to produce a skin response and b) analyzing the skin of said transgenic mice to determine the reduction of skin response, wherein the reduction of skin response indicates that the composition, compound, or procedure can reduce skin response.

20 In one embodiment of the method, the composition or compound can be administered orally or by injection.

25 In another embodiment of the method, the composition or compound can be administered topically by contacting the composition or compound with the skin of the transgenic mice.

30 In another embodiment of the method, the procedure is not previously known.

In another embodiment of the method, the procedure is identified by the method.

35 In another embodiment of the method, the procedure is DNA vaccination.

In another embodiment of the method, the composition or compound is not previously known.

5 In another embodiment of the method, the composition or compound is identified by the method.

In another embodiment of the method, a mixture is produced for reducing skin response comprising an effective amount of the composition or compound identified by the method and 10 a suitable carrier.

In another embodiment of the method, the skin response is inflammation, tanning, skin carcinoma, melanoma or hyperpigmentation.

15 In another embodiment of the method, the hyperpigmentation is natural occurring hyperpigmentation or post inflammatory hyperpigmentation.

20 In another embodiment of the method, the inflammation is associated with human hyperpigmentation, or human hypopigmentation.

25 In another embodiment of the method, the subject is a mouse or a human-being.

In another embodiment of the method, the epidermal stem cell factor transgene encodes either a membrane bound epidermal stem cell factor or a membrane/soluble epidermal 30 stem cell factor.

In another embodiment of the method, the epidermal stem cell factor transgene encodes a membrane or soluble epidermal stem cell factor.

35 In another embodiment of the method, the epidermal stem cell factor transgene is cloned into a construct containing

a human cytokeratin 14 promotor.

5 In another embodiment of the method, the human cytokeratin 14 promotor causes the expression of the stem cell factor transgene in murine skin of the basal layers of the interadnexal epidermis and the follicular epithelium.

10 In another embodiment of the method, the skin response of the transgenic mice can be induced by applying an irritant or an allergic dermatitis inducing agent to said skin.

In another embodiment of the method, the irritant is croton oil or dinitrofluorobenzene.

15 In another embodiment of the method, the croton oil or dinitrofluorobenzene are applied to the ear or the abdominal skin of the transgenic mice; wherein the abdominal skin is either hairless or shaved.

20 In another embodiment of the method, the croton oil is used at a concentration of 0.2 percent.

25 In another embodiment of the method, the dinitrofluorobenzene is used at a concentration of 0.5 percent in a 4:1 mixture of acetone and olive oil.

In another embodiment of the method, the reduction or treatment of hyperpigmentation is determined by electron microscopic analysis.

30 In another embodiment of the method, the compound is an epidermal stem cell factor inhibitor.

35 In yet another embodiment of the method, the stem cell factor inhibitor is a monoclonal antibody.

In a further embodiment of the method, the monoclonal

antibody is ACK2.

The present invention further provides a method of identifying a composition, a compound or a procedure which 5 can reduce radiation damage to the skin of a subject, comprising: a) administering said composition or compound, or applying said procedure to the transgenic mice which express endogenous epidermal stem cell factor, b) subjecting the skin of said transgenic mice and the skin 10 of the control transgenic mice to radiation, and c) analyzing the effects of said composition, compound, or procedure on reducing skin radiation damages.

In one embodiment of the method, the composition or 15 compound can be administered orally or by injection.

In another embodiment of the method, the composition or compound can be administered topically by contacting the composition or compound with the skin of the transgenic 20 mice.

In another embodiment of the method, the procedure is not previously known.

25 In another embodiment of the method, the procedure is identified by the method.

In another embodiment of the method, the procedure is DNA 30 vaccination.

In another embodiment of the method, the composition or compound is not previously known.

35 In another embodiment of the method, the composition or compound is identified by the method.

In another embodiment of the method, a mixture is produced

for reducing skin radiation damages comprising an effective amount of the composition or compound identified by the method and a suitable carrier.

5 In yet another embodiment of the method, the radiation is ultra-violet light.

10 In a further embodiment of the method, the radiation damage is tanning, carcinogenesis, photo-aging, photo-damage or the development of melanoma.

15 The present invention also provides a pharmaceutical composition for treating human skin diseases, comprising (a) a compound that can treat skin diseases of the transgenic mice which express endogenous epidermal stem cell factor, and (b) a suitable carrier, wherein the compound specifically targets the epidermal stem cell factor or its receptor.

20 In one embodiment of the pharmaceutical composition, the compound is ACK2.

25 This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Transgene construction: Two murine SCF cDNAs were cloned into constructs containing the human cytokeratin 14 upstream region (27) (Figure 1). This promoter causes expression in the skin limited to the basal layers of the interadnexal epidermis and the follicular epithelium. The cDNAs were both full length clones, containing exon 6 encoded sequences. One cDNA (transgene one) was unmodified and therefore could produce a membrane-bound protein with the exon 6 encoded protease sensitive site, from which a soluble, bioactive form of SCF could be efficiently generated (10, 11, 28). The product of this transgene will be referred to as membrane/soluble SCF. The second cDNA (transgene two) had been previously modified by site directed mutagenesis, deleting the primary high efficiency cleavage site (between amino acids 164 and 165) and an alternate exon 7 encoded low efficiency cleavage site (found in murine SCF between amino acids 180 and 181). The SCF produced by this transgene therefore exists predominantly as a membrane-bound molecule (membrane SCF) (29). Both cDNAs have been previously shown to produce biologically active SCF (29, 30).

Generation and analysis of transgenic animals: Two μ g/ml transgenic DNA, in 10 mM Tris (pH 7.5), 0.1 mM EDTA was injected into fertilized oocytes collected from pseudopregnant mice as described (31). At birth, most transgene expressing mice could be identified by distinctive pigmentary phenotypes, as described in the Results section. Integration of transgenes was verified by polymerase chain reaction (PCR) of genomic DNA with transgene-specific primers and copy number estimated by Southern blotting of PCR products, followed by autoradiography and densitometry. Skin specific expression of transgene messenger RNA was confirmed by northern blotting and by reverse transcription-polymerase chain

reaction with transgene specific primers using RNA extracted from representative animals. Transgene expression was quantitated by RPA II Ribonuclease Protection Assay Kit (Ambion, Austin, TX, USA) according to the manufacturer's directions. Briefly, total RNA extracted from mouse skin was hybridized with digoxigenin labeled single stranded RNA probes for twenty three hours at 42°C, digested with RNase A and RNase T1, electrophoresed through 5% polyacrylamide/7 Molar urea, protected fragments were transferred to Hy⁺ membrane (Boerheringer-Mannheim, Indianapolis, IN, USA), bands detected by chemiluminescences, and band density determined by densitometry. Preliminary studies of RNA preparations from each transgenic line were performed to measure Beta-actin, and the amounts of RNA for SCF mRNA determinations adjusted for comparison. RNA was also used with reverse transcription and the polymerase chain reaction for direct amplimer sequencing of c-kit mRNA sequences in regions which could contain known activating mutations, as previously described (25). The primers used were 5' CAAATC/GCATCCC/TCACACCCTGTTCAC and 5' CCATAAGCAGTTGCCTCAAC which bind to nucleotides 1568→1593 and 1854→1835 and 5' TGTATTCACAGAGACTTGGC and 5' AAAATCCCATAGGACCAGAC binding to nucleotides 2384→2403 and 2595→2576. These regions contain the codons with both of the activating mutations, codon 559 and codon 814, respectively which have been described in human mastocytosis and in a murine mast cell line (5, 26).

Transgene one, containing the full-length unmodified SCF cDNA (membrane/soluble SCF), was injected into 100 F1 oocytes (C57 BL6 x SLJ), which were implanted into six host mothers, resulting in four independent hyperpigmented mice, all of which were positive for the transgene, and 40 other littermates which were pigmentary phenotype negative and transgene negative by PCR.

Oocytes for transgene two (membrane SCF) were F1 (C57BL/6J

female x SLJ/J male), and the offspring could be black, agouti, or white. Injection of 40 embryos and implantation into six host mothers generated 48 pups, 21 of which were positive for integration by PCR. Of the 25 founder mice 5 identified by PCR with the transgene specific primers, 3 were black, 13 were agouti, and 9 were white. Five PCR positive mice (3 agouti and 2 black) showed a clearly identifiable pigmentary phenotype. Given the inability of white mice to produce normal cutaneous pigment, it is 10 possible that there were also white founders that expressed the transgene without the production of an obvious change in pigment. Backcrossing of phenotype positive, black and agouti founders to C57 BL/6 mice produced uniform pigmentary changes, described in the Results section.

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Histology, immunohistochemistry, and electron microscopy: Tissues from transgenic and littermate mice were fixed in formalin and embedded in paraffin or polyester wax, sectioned, and stained with hematoxylin and eosin, azure 20 blue, alcian blue, or Giemsa's stain according to standard techniques (31-33). Immunofluorescence studies were performed on polyester wax embedded sections or frozen sections, also using standard techniques. Antibodies included anti-S100 (rabbit anti-cow S100, pre-diluted, 25 Dako, Carpinteria, CA), and the ACK2 and ACK4 monoclonals (rat anti-mouse c-kit (34), at 20 µg/ml). Controls included omission of the primary antibody or the use of isotype matched monoclonal antibodies of irrelevant specificity. Electron microscopy was done as previously 30 described (35).

Inflammation Inducement and Treatment

We used Croton Oil and dinitrofluorobenzene (DNFB), respectively to reduce irritant and allergic contact 35 dermatitis, respectively, in HK14-SCF transgenic mice and their non-transgenic litter-mates. Croton Oil was applied directly to the ears of mice and DNFB was applied to the

ears of mice after sensitization on shaved abdominal skin. Ear swelling was measured with a micrometer. In the ear-swelling test, the transgenic mice were divided into two groups; one group was treated with the murine 5 monoclonal antibody ACK2, which blocks the interaction of SCF with its receptor (KIT), and the other group was treated with only saline. In addition, shaved abdominal skin of some mice was also treated with Croton Oil or with DNFB, and observed for inflammation and hyperpigmentation.

10 For statistical analysis, we used a mixed effects model, which allows us to fit repeated measurements over time and to compare different groups over time. We also performed orthogonal contrasts to evaluate the difference between 15 treated and control groups at each time point. Immunoperoxidase study, using anti human SCF monoclonal antibodies, were performed in skin by standard method (7).

Experimental Results

20 Dermal mast cells accumulate in the presence of membrane/soluble keratinocyte SCF. Sections of skin from all mice producing membrane/soluble SCF (transgene one) showed increased mast cells in the dermis (Figure 2). In newborn transgene one positive mice, the mast cells were 25 superficial near the dermal-epidermal junction, close to the epidermal source of soluble SCF (Figure 2a). In older mice the mast cells filled the papillary dermis in some areas, but were also present in the reticular dermis, in a pattern identical to that of human mastocytosis (Figure 30 2, b-d). Electron microscopic analysis confirmed the presence of numerous mast cells with characteristic granules within the dermis of the transgene one positive animals, and also showed that some of the heavily pigmented 35 cells within the dermis of transgene one positive mice were melanocytes (Figure 3). Mast cells were relatively rare and dermal melanocytes were not detected in the body wall skin of non-transgenic littermates and in transgene two

positive animals of equivalent age. These observations were true across a wide range of copy numbers and levels of SCF mRNA expression (Figure 4). Since the keratin 14 promoter is properly expressed in the skin only by keratinocytes, and since the production of only membrane-bound keratinocyte SCF did not spontaneously result in increased dermal mast cells in transgene two positive animals, keratinocyte production of the soluble form of SCF appears to be able to cause cutaneous mastocytosis in mice.

SCF transgenic mice are hyperpigmented: Targeted expression of each of the SCF transgenes in murine skin caused a similar, distinctive pigmented phenotype. The pigment responsible for the coat color of normal mice resides in the hair follicles and hair shafts, not in the epidermis. The transgenic mice, however, developed prominent epidermal pigmentation (Figure 5). Transgene positive animals could be identified by increased pigment at birth. By approximately 21 days of age, the phenotypes were well established; phenotype positive animals showed pigmentation of most of the skin as well as increased pigmentation of most of their skin as well as coat pigment. Extensive pigmentation was noted in a number of areas including the nose, mouth, ears, paws, and external genitalia when compared to normal littermate controls. There was enough individual variation in pigmentation so that no clear correlation between the level of pigmentation and the levels of transgenic expression could be shown. All transgenic animals showed similar degrees of pigmentation regardless of transgene type, copy number, or levels of SCF mRNA expression. In addition to the epidermal pigmentation, the three transgene two positive agouti founders showed thin black transverse strips, consistent with the pigment distribution of the allophenic mice described by Beatrice Mintz (pictures not shown) (36).

Numerous melanocytes are maintained in the skin of transgenic mice: The increased pigmentation of the skin of the transgene positive mice of both types is attributable to the presence of intraepidermal melanocytes, 5 and to the epidermal melanin produced by those cells. Intraepidermal melanocytes can be identified in hematoxylin and eosin stained sections as cells in the basilar layers surrounded by clear halos (Figure 6, a & b) or in immunoperoxidase preparations by their expression of S-100 10 protein. Immunohistochemical analysis of animals expressing each of the transgenes showed numerous S-100(+) intraepidermal melanocytes (please also see Figure 2a). These melanocytes can be differentiated from Langerhans 15 cells, which also express S-100 protein, because melanocytes are in the basal layers and Langerhans are in the suprabasal layers. Melanocytes can also be differentiated from Langerhans cells by their expression of the kit protein, the receptor for SCF, which is not expressed by Langerhans cells. Staining of transgenic 20 animal skin with anti-kit antibody identified well-developed dendritic cells within the basilar layers of the epidermis and follicular epithelium, consistent with melanocytes (Figure 6c and 2b).

25 Histologic examination confirmed the presence of pigment within the epidermis of both transgene one and transgene two phenotype positive mice from all sites examined, including the ears, tail, footpads, and body wall (Figure 6a). In addition, transgene one positive mice showed many 30 pigmented cells within the dermis. Pigmentary abnormalities were not observed in transgene negative littermates. Only slight epidermal pigment was identified in these control mice, and mostly in non-hair bearing areas like the footpad and tail. Although pigment patterns were stable throughout 35 much of the adult life of the mice, an occasional TG1 (msSCF) mouse developed patchy areas of depigmentation, mostly in the ears, associated with loss of epidermal

melanocytes and increased pigment incontinence. This phenomenon was not observed in the mSCF mice.

5 Electron microscopy confirmed the presence of numerous melanocytes within the epidermis of both types of transgenic mice (Figure 7). Pigmented keratinocytes, similar to those seen in the epidermis of humans, were also present in the interadnexal epidermis of the transgenic mice. Intraepidermal melanocytes and pigmented keratinocytes were extremely rare in control mice.
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15 All transgenic mice showed ear swelling which was greater in magnitude and more prolonged than the non-transgenic (B6) control mice: Blocking SCF by administration of ACK2 decreased the magnitude of ear swelling in transgenic mice, as shown in the following figure 8.

20 Averaging across time: There is a difference between the ACK2 treated and the control saline treated transgenic mice, which is significant at the 0.05 level. Averaging across time, there is also a significant difference between each of the two groups of transgenic mice (ACK2 treated and control) and the non-transgenic mice. Both comparisons are statistically significant at the 0.0001 level. See Figure 25 8. Observation of the abdominal wall skin treated with Croton Oil or DNFB showed hyperpigmentation and thickening which was not observed in non-transgenic mice control (B6) mice that were treated identically. Histologically, hyperpigmentation correlated with dermal melanophages and 30 increased epidermal melanin, identical to the changes seen in human postinflammatory hyperpigmentation.

Discussion

35 Melanocytes are maintained in human epidermis throughout life. In normal mice DOPA reaction positive cells (melanoblasts and melanocytes) are found in the epidermis at birth, but their number decreases from postnatal day 4

and is severely reduced after one month of age (37). One possible explanation for the maintenance of epidermal melanocytes in human skin, and the difference between the distribution of melanocytes in adult human and murine skin, 5 could be expression of epidermal SCF. Human epidermal keratinocytes produce SCF (7, 8, 39), but the SCF gene does not appear to be expressed in murine epidermis (9). The results presented here show that SCF expression by murine epidermal keratinocytes causes the maintenance and 10 stimulation of epidermal melanocytes throughout life. These data support the hypothesis that the decrease in melanocyte numbers in the postnatal mouse epidermis is due to a lack of local SCF expression. In combination with the 15 fact that the soluble SCF produced by S1/Sld mice is insufficient to support normal melanocyte survival and the observations that membrane-bound SCF promotes longer lasting kit activation and increased survival of kit dependent cells in the hematopoietic system (40, 41), our data suggest that it is specifically the membrane-bound 20 form of SCF that is crucial for melanocyte survival and function.

It is interesting to note that none of the animals expressing either of the transgenes described in this paper 25 have developed melanoma to date, a finding which supports previous observations that stimulation of the kit tyrosine kinase receptor does not appear to promote the development of melanocytic tumors (40). It also seems likely that the animals described herein, or animals derived from them, 30 will be useful in the study of cutaneous mastocytosis and epidermal melanocyte biology.

The fact that SCF transgenic mice have greater responses to allergic and irritant contactants shows that epidermal 35 SCF can actively contribute to eczematous dermatitis. This interpretation is confirmed by our demonstration that the inflammation can be diminished by blocking the SCF receptor

with the ACK2 monoclonal antibody. Since human post natal epidermal keratinocytes express SCF, unlike post natal murine epidermal keratinocytes, and alterations of human epidermal SCF are found in spongiotic dermatitis (a form of eczema), these observations also support our contention that the skin of mice expressing epidermal SCF is a better model of human skin than is the skin of normal mice. Further supporting this claim is our previous observation of increased soluble epidermal SCF in the hyperpigmented lesions of mastocytosis. In sum, these data support our claim that animals expressing epidermal SCF are more suitable for a wide variety of investigations than those which do not.

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